TREHALOSE-ASSISTED PRECIPITATION IN THE SYNTHESIS OF LACTASE CROSS-LINKED ENZYME AGGREGATES

A Project Paper

Presented to the Faculty of the Graduate School

of Cornell University

in Partial Fulfillment of the Requirements for the Degree of

Master of Professional Studies in Agriculture and Life Sciences

Field of Food Science

by

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August 2018

ABSTRACT

Free lactase is widely used in the dairy industry to produce lactose-free products, but it is usually expensive, has low stability and cannot be reused. Immobilized enzymes are often used to overcome the drawbacks of using free enzymes. Cross-linked enzyme aggregates (CLEAs), a carrier-free immobilization technique, was performed in this study. Lactase enzymes were precipitated by an organic solvent (acetone) and cross-linked with a cross-linking agent (glutaraldehyde) to produce CLEAs. Trehalose was integrated into the lactase CLEAs to potentially help stabilize the protein structure and thus improve the activity retention of the immobilized enzyme. It was found that no significant results exist between the activity of the lactase CLEAs alone to the lactase CLEAs with trehalose. Stabilizing lactase CLEAs with additives has potential to improve the immobilize enzyme activity, but further research and optimization in the immobilization methods is required to achieve statistically significant results. Upon successful immobilization of the enzymes and improved activity retention, the immobilized lactase CLEAs will have potential uses in the dairy industry to produce lactose-free products at reduced costs.

Key words: enzyme, lactase, dairy, immobilization, Cross-linked enzyme aggregates (CLEAs)

BIOGRAPHICAL SKETCH

Zhixin was born in China, but grew up in Montreal, Canada where she completed her Bachelor of Science in Food Chemistry at McGill University (17'). She then came to Cornell University to pursue a MPS degree in Dr. Julie Goddard's lab, working on the immobilization of the enzyme lactase. Zhixin enjoys facing new challenges and has a passion for food. In her bare times, she loves to experiment with new recipes and food fermentation.

I would like to dedicate this work to my dear mother, father and younger brother.

Je voudrais dédier ce travail à ma mère, mon père et mon petit frère.

ACKNOWLEDGMENTS

Deepest thanks to my advisor, Dr. Julie Goddard and my lab mates for their help and support. Without their support, it would not have been possible for me to realize this project.

First and foremost, I would like to express my deepest appreciation to my advisor Dr. Julie Goddard, without whom this work would not have been possible.

Secondly, I would like to thank Dr. Stephanie Andler for her patience, help and guidance throughout this project.

Then, I would like to thank Ms. Brenda, for providing me training on laboratory safety and instruments usage.

In addition, I would like to applaud all my lab mates: Pei, Jason, Danhui, Hannah, Josh, Troy, and Emma for their support.

Lastly, I would like to thank my family and friends for their words of encouragement throughout this journey.

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LIST OF ABBREVIATIONS

CLEAs: Cross-linked enzyme aggregates

ONPG: Ortho-nitrophenol-ß-galactopyranoside

SEM: Scanning electron microscopy

INTRODUCTION

Approximately 65% of the world population has a reduced ability to digest lactose, a disaccharide found in dairy products (NIH, 2018). In the dairy industry, lactase is the enzyme responsible for the breakdown of lactose into glucose and galactose, producing lactose-free dairy products targeting consumers with lactose intolerance. However, the use of free enzymes is usually expensive and unstable to thermal and pH fluctuations. Enzyme immobilization is a method to improve the stability of these proteins over a wider range of temperature and pH environments.

Methods of immobilization include adsorption or covalent binding of the free enzymes onto a surface and entrapment or encapsulation of the enzymes into liposomes (Allison & Bering, 1998; Mohamad, Marzuki, Buang, Huyop, & Wahab, 2015). On the other hand, a carrier-free immobilization method exists to immobilize enzymes via cross-linking using a cross-linking agent to produce cross-link enzyme aggregates (CLEAs) (Sheldon, 2007). This technique is usually cheap and results in a cluster of enzymes that are reusable and more stable than free enzymes (Li et al., 2015). The most commonly used cross-linking agent is glutaraldehyde (Barbosa et al., 2014).

Sheldon's group obtained promising results of CLEAs with different enzymes including penicillin G amidase (Cao, Rantwijk, & Sheldon, 2000) and lipases (Lopez-Serrano, Cao, Rantwijk, & Sheldon, 2002). On the other hand, another group prepared CLEAs from β-galactosidase and successfully scaled-up the process (Schoevaart et al., 2004).

Past studies have shown an increase interest towards CLEAs and their potential uses in the industrial settings. However, despite their advantages over free enzymes and their cost-effective

preparation methods, cross-linking is far from being the perfect immobilization method. CLEAs synthesis can be separated into two main steps: precipitation with the help of an organic solvent and cross-linking with the help of a cross-linking agent. One major drawback in the preparation of CLEAs lies the precipitation step. In fact, precipitation using organic solvents during the precipitation step can result in some degree of denaturation of the enzyme, resulting in activity loss and thus a lower activity yield of the final CLEAs (Wang et al., 2011).

Trehalose, a disaccharide of two glucose molecules, is a food additive that is generally regarded as safe (GRAS). Studies on trehalose suggest that this sugar might protect proteins from denaturation during extreme conditions (Jain & Roy, 2009). Wang et al. (2011) studied the effect of different sugars, including glucose, sucrose and trehalose, on CLEAs. They concluded from their results that CLEAs with the highest activity were obtained from the addition of trehalose at 20% (w/v).

There are three main theories to explain the properties of trehalose: vitrification, preferential exclusion and water replacement. The first theory proposes that trehalose forms an external matrix around the protein and physically protects it from external stress. On the other hand, the preferential exclusion suggests that no direct interaction exists between the protein and the sugar. Instead, trehalose would act on the water molecules to move them away from the protein. Lastly, the water replacement theory explains that trehalose replaces the hydrogen bonds from the water that might be stripped-off during denaturation condition, helping the protein to keep its stability (Jain & Roy, 2009).

OBJECTIVE

The overall goal of this project was to stabilize and improve the activity of the CLEAs by incorporating trehalose in the enzymes aggregates. Trehalose is known to stabilize protein structures and has shown efficacy against protecting enzyme (Jain & Roy, 2009), but hasn't been studied with lactase CLEAs previously. The lactase CLEAs were produced using acetone as a precipitant and glutaraldehyde as cross-linking agent. It was expected that trehalose would help improve the activity and structure of the lactase CLEAs.

MATERIAL AND METHODOLOGY

1. Determination of protein in lactase powder

Prior to use, the protein concentration in the lactase sample was determined. Lactase powder from aspergillus oryzae was dissolved in potassium phosphate buffer (pH 6.8) and purified by filtration through a 0.22um pore size filter syringe and analysed spectrophotometrically using BCA routine measurement for proteins at 280nm.

2. Purification of enzyme

The protein content in the lactase powder was determined to be 21%. Accordingly, 476.2mg of lactase powder was dissolved into 10ml potassium phosphate buffer (0.1M, pH 6.8) to make a final concentration of 10mg/ml of lactase solution. The lactase-buffer solution was then passed through a 0.22um pore size syringe filter. The filtered lactase solution was separated into 450ul and pipetted into centrifugation filtration tubes to be centrifuged for 15 min at 14000 G-force. 270ul of potassium phosphate buffer was added back to the tubes and the solution was pipetted out into a clean 5ml tube. The final purified protein solution was then tested for protein content at 500ug/ml using the BCA protein assay.

3. BCA Lactase Standard Curve

2ml of 500ug/ml lactase solution was prepared by dissolving 19mg of unpurified lactase powder in 2ml potassium phosphate buffer. Nine 1.5ml vials were labelled from A-I. Following table 1. the right amount of buffer and lactase solution was added into each tube and the tubes were vortexed. 300ul of each tube was transferred into a 96-well UV microplate and the absorbance was

measured with the BIOTEK at 280nm. The standard curve was obtained by plotting the protein concentration (mg/ml) versus the absorbance (280nm).

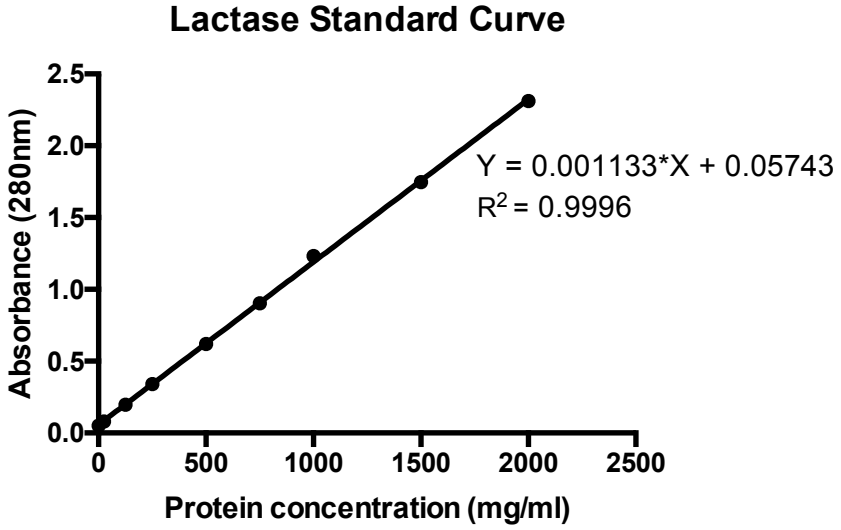

Figure 1. Example of a Lactase Standard Curve

Table 1. BCA Lactase Standard Curve

Vial	Volume of buffer (ul)	Volume lactase (500ug/ml)	Concentration (ug/ml)
A	0	300	2000
B	125	375	1500
C	325	325	1000
D	175	175 of B	750
E	325	325 of C	500
F	325	325 of E	250
G	325	325 of F	125
H	400	100 of G	25
I	400	0	0

4. Test for protein content at 500ug/ml

25ul of the purified lactase solution was added to 475ul of potassium phosphate buffer. 300ul of this diluted lactase solution was then pipetted into a UV 96-well microplate and absorbance was measured at 280nm, along with the lactase standard curve samples. The protein concentration was determined with the BCA lactase standard curve.

5. Stock solution of trehalose in phosphate buffer

Starting with 5ml of 8mg/ml of lactase solution, X-ml of buffer was needed to be added into 5ml of buffer solution to obtain a final solution of 7mg/ml lactase in buffer.

X-ml = $(\frac{5ml*8mg/ml}{7mg/ml})$ – 5ml = 714.3ml

The final volume is then 5.7143ml

20% (w/v) of trehalose is used, which is 1.14mg

To prepare the stock, the proportion of 1.14mg of trehalose in 714.4ml of buffer is used. Then, 714.3ml of trehalose-buffer stock solution is added in 5ml of 8mg/ml of lactase solution to get a final concentration of 7mg/ml with 20% (w/v) of trehalose.

6. CLEAs formation with acetone and glutaraldehyde

Eight 2ml vials were labelled from 1 to 8. Following table 2, the purified lactase solution was first added. Acetone was then slowly added, dropwise, into each tube and allowed to mix for 30 minutes. Subsequently, glutaraldehyde was added at a final concentration of 93.4mM, vortexed and allowed to react overnight for 22 hours (continuous mixing). Following cross-linking, the CLEAs were recovered by centrifuging the mixture for 1 minute at 10 000 G-force. The supernatant was then decanted and the precipitate, containing the CLEAs, was washed trice with

potassium phosphate buffer, centrifuging 1 min at 10 000 G-force between each wash. Finally, the recovered CLEAs were stored in 320ul of potassium phosphate buffer prior to activity measurements.

Table 2. Lactase CLEAs Preparation

Samples	Purified lactase solution in phosphate buffer	Trehalose (ug)	Acetone (ul)	gluteraldehyde (50%) (ul)	Cross-linking time (hours)
1	320ul of 7mg/ml	64	1280	28.8	Overnight (22hrs)
2	320ul of 7mg/ml	64	1280	28.8	Overnight (22hrs)
3	320ul of 7mg/ml	64	1280	28.8	Overnight (22hrs)
4	320ul of 7mg/ml	64	1280	28.8	Overnight (22hrs)
5	320ul of 7mg/ml	0	1280	28.8	Overnight (22hrs)
6	320ul of 7mg/ml	0	1280	28.8	Overnight (22hrs)
7	320ul of 7mg/ml	0	1280	28.8	Overnight (22hrs)
8	320ul of 7mg/ml	0	1280	28.8	Overnight (22hrs)

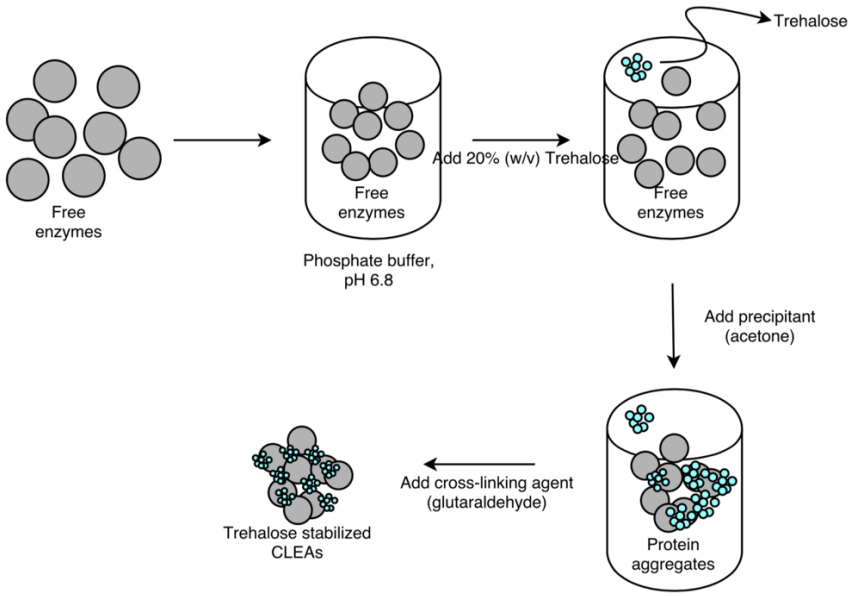

Figure 2. Proposed methodology of trehalose stabilized lactase CLEAs, adapted from (Barbosa et al., 2014)

7. Microplate ONPG Assay – Activity Assay (pH and temperature studies)

The immobilized lactase activity was analyzed spectrophotometrically in a BIOTEK using ONPG. The method to analyze lactase activity was adapted from Goddard and Talbert (Goddard & Talbert, 2006). Material used included deionized water, ONPG, and potassium phosphate buffer (pH 6.8). A 0.5 mg/ml solution of ONPG in buffer was prepared by dissolving 10mg ONPG in 20mL 0.1M Potassium Phosphate Buffer, pH 6.8.

Activity of the CLEAs was evaluated at pH 4, pH 6.8 and pH 10. A sodium citrate buffer solution was prepared at a pH 4 and a sodium bicarbonate buffer solution was prepared at a pH 10. To evaluate the CLEAs at different pHs, 42ul of the stored CLEAs was pipetted into 219.6ul of the respective buffer solution and incubated at 4^{o}C for 24 hours in 1.5ml tubes. To study the effect of temperature on CLEAs, 42ul of the immobilized enzymes were stored at 4^{o}C, 21^{o}C and 37^{o}C for 24 hours in 219.6ul of potassium phosphate buffer (pH 6.8). Then, to test the activity following incubation, 87.2ul of each sample was transferred in a well of a 96-well microplate. Using a multi-channel pipette, 211.2ul of the ONPG solution was pipetted in each well and tested at 420nm in the BIOTEK.

8. ONP Standard Curve

From the ONP standard curve (example shown in figure 3), the extinction coefficient was obtained to calculate the activity. The activity in millimolar of ONP per second is obtained by dividing the slope of the linear portion of the CLEAs absorbance at 420 nm over time curve by the extinction coefficient.

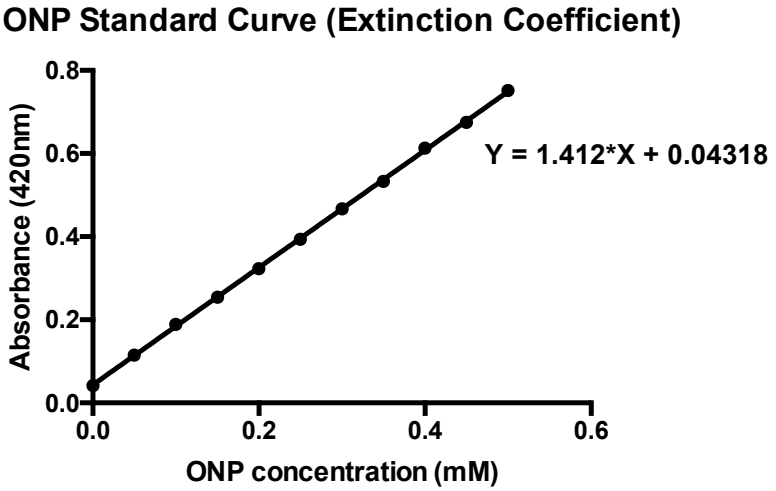

Figure 3. ONP Standard Curve (Extinction Coefficient)

Enzyme activity = $\frac{\frac{Absorbance\ (420nm)}{time\ (sec.)}}{\frac{Absorbance\ (420nm)}{ONP\ (mM)}} = \frac{ONP\ (mM)}{time\ (sec.)}$ = Slope of the ONP standard curve

9. SEM imaging

SEM imaging of the lactase CLEAs at different magnifications showed its surface morphology. SEM imaging of CLEAs with and without trehalose were compared and analysed. In fact, the morphology of the CLEAs is important as it influences the mass transfer and filterability of the solution when applied in industry settings (Talekar et al., 2013).

10ul of the sample to be tested was pipetted onto a piece of aluminum foil. The foil was placed in a glass petri dish and allowed to air dry in the fume-hood overnight. The sample was then sputter coated and tested for SEM.

RESULTS AND DISCUSSION

Conventional CLEAs synthesis does not require initial purification of the enzyme. However, for the purpose of this study, to evaluate the effect of trehalose, the initial lactase powder was purified to exclude other bulking agents from the enzyme solution, including sugars other than trehalose, that could have potentially interfered with the results.

In this work, 20% (w/v) of trehalose was mixed in the initial purified enzyme solution to potentially protect the protein from excess dehydration during the precipitation with organic solvents (Wang et al., 2011). The enzymes were then precipitated with acetone and glutaraldehyde (93.4mM) was used as a cross-linking agent to form CLEAs. Due to a large difference in activity between the free lactase and the lactase CLEAs, free lactase activity was not incorporated into the graphical representation (Figure 4). In fact, the main objective was to study the effect of trehalose on the lactase CLEAs' activity. The activities of the sugar-free CLEAs and CLEAs with trehalose were measured at different pHs and temperatures (Figure 4). From the results, the average activity of CLEAs with trehalose was higher than the sugar-free CLEAs in all conditions. However, the high standard deviation in the measured activities suggests that the differences are not statistically significant for a conclusion to be made. An observation from the experiment that would explain this variation in activity measurements includes non-uniform size of the synthesized CLEA particles. The starting concentration, choice of precipitating agent, cross-linking agent as well as reaction time and agitation speed can all influence the size of the CLEAs. It was reported that the enzyme and cross-linking agent concentration played a major role in the final particle size and shape (Talekar et al., 2013; Velasco-Lozano, López-Gallego, Mateos-Díaz, & Favela-Torres, 2016). Then, previous work on enzyme CLEAs found that the degree of centrifugation to recover

the immobilized enzymes might have an impact on the size of the particles (Schoevaart et al., 2004). Optimizing CLEA synthesis in the future to obtain uniform sized CLEAs would likely result in a more consistent activity measurement.

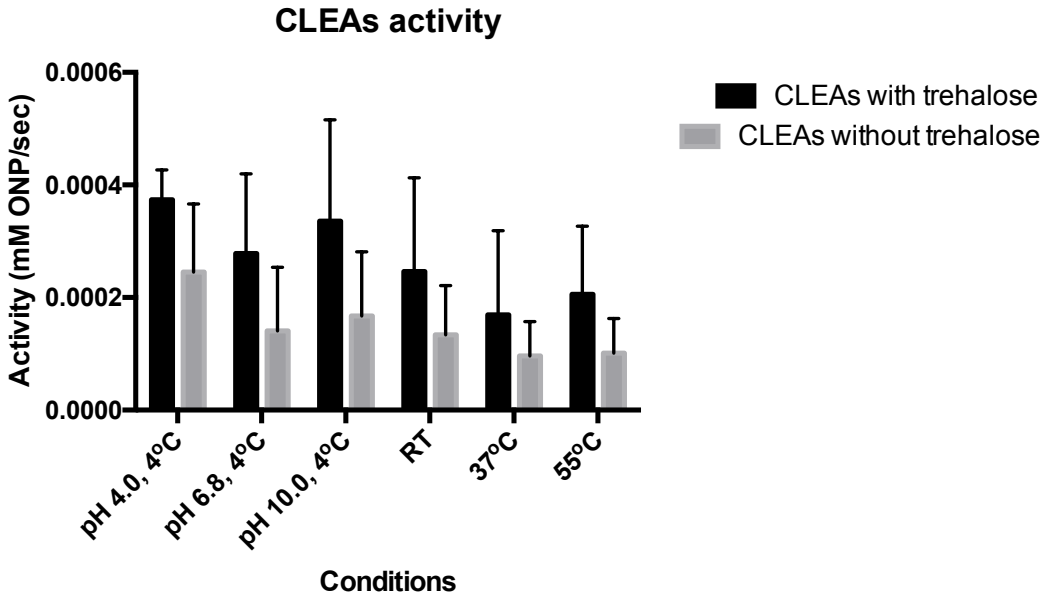

Figure 4. CLEAs activity under different pH and temperature conditions

In the study of the effect of trehalose on CLEA particle size, Wang et al. reported that the addition of trehalose resulted in larger CLEAs (Wang et al., 2011). They associated this effect to the hydrogen-bond, acting as a water-substitute between the trehalose and the enzyme. Due to the high stability of trehalose, its immiscibility in organic solvent and the bond that it formed with the enzyme, it resulted in larger aggregates. On the other hand, they also explained that organic solvents likely increase the electrostatic attraction between the enzyme molecules, which would result in the proteins packing more tightly together, giving rise to smaller size CLEAs. Since trehalose would act as a protectant, the electrostatic attraction would less likely to increase drastically, preventing the enzymes to pack extensively and lose activity.

Figure 5a, represents an SEM image of lactase CLEAs with trehalose at x1000 magnification. Nonuniform size and shape of lactase CLEAs were observed in both sugar-free lactase CLEAs (figure 6) and CLEAs prepared with trehalose (figure 5a-b), which might have affected activity measurement. As mentioned previously, these clusters of CLEAs were reported to be due to extensive centrifugation in the recovery step (Schoevaart et al., 2004). Nonetheless, the lactase CLEAs showed a spherical shape with size varying between 1-5 µm. It is difficult to compare to existing literature, as small changes in the synthesis condition of the immobilized enzyme can affect the morphology of the resulting CLEAs (Schoevaart et al., 2004).

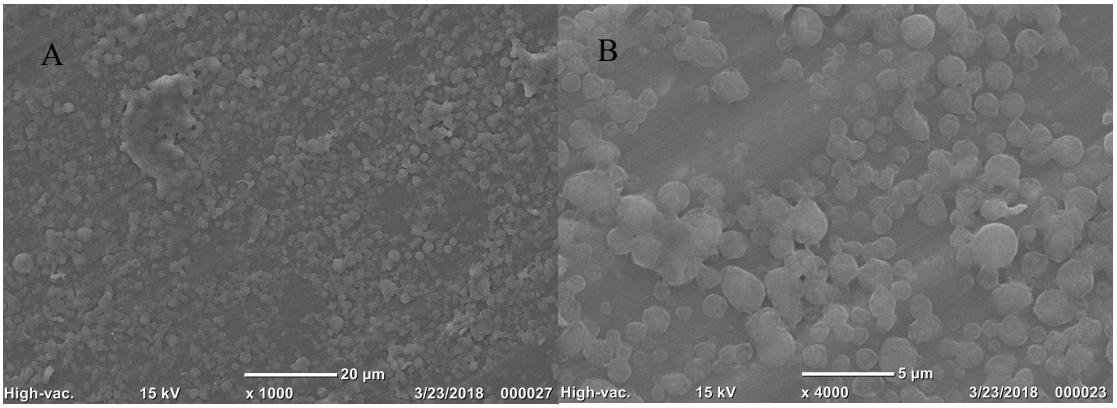

Figure 5. Scanning electron microscope images of lactase CLEAs with trehalose at (A) magnification X1000; (B) magnification X4000

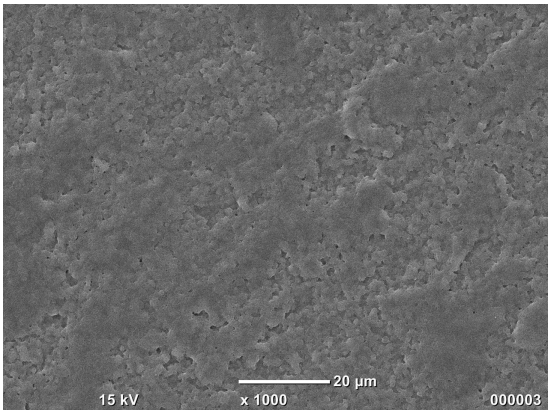

Figure 6. Scanning electron microscope images of lactase CLEAs without trehalose at magnification X1000.

CONCLUSION

In conclusion, lactase Cross-Linked Enzymes Aggregates were synthesized using acetone as the precipitant and glyceraldehyde as the cross-linking agent. Trehalose was added during the precipitation step to potentially improve the activity of the final cross-linked enzyme aggregates. On average, a slight improvement in the activity of CLEAs with trehalose was seen for both temperature and pH studies compared to the sugar-free CLEAs. However, looking at the standard deviations, statistical analysis concluded that the differences were not significant. Future work should look into further optimizing the lactase CLEAs synthesis conditions, including pH, temperature, cross-linking time and agitation speed, protein concentration and precipitant concentration.

WORK CITED

Allison, M. J., & Bering, C. L. (1998). Immobilized Lactase in the Biochemistry Laboratory. *J. Chem. Educ., 75*(10), 1278. doi:10.1021/ed075p1278

Barbosa, O., Ortiz, C., Berenguer-Murcia, Á., Torres, R., Rodrigues, R. C., & Fernandez-Lafuente, R. (2014). Glutaraldehyde in bio-catalysts design: a useful crosslinker and a versatile tool in enzyme immobilization. *RSC Adv., 4*(4), 1583-1600. doi:10.1039/c3ra45991h

Cao, L., Rantwijk, F. v., & Sheldon, R. A. (2000). Cross-Linked Enzyme Aggregates: A Simple and Effective Method for the Immobilization of Penicillin Acylase. *Organic letters, 2*(10), 1361-1364.

Goddard, J., & Talbert, J. (2006). *Lactase Activity Measurement* . Food Science. Cornell University.

Jain, N. K., & Roy, I. (2009). Effect of trehalose on protein structure. *Protein Sci, 18*(1), 24-36. doi:10.1002/pro.3

Li, L., Li, G., Cao, L. C., Ren, G. H., Kong, W., Wang, S. D., . . . Liu, Y. H. (2015). Characterization of the cross-linked enzyme aggregates of a novel beta-galactosidase, a potential catalyst for the synthesis of galacto-oligosaccharides. *J Agric Food Chem, 63*(3), 894-901. doi:10.1021/jf504473k

Lopez-Serrano, P., Cao, L., Rantwijk, F. v., & Sheldon, R. A. (2002). Cross-linked enzyme aggregates with enhanced activity: application to lipases. *Biotechnology Letters, 24*, 1379-1383.

Mohamad, N. R., Marzuki, N. H., Buang, N. A., Huyop, F., & Wahab, R. A. (2015). An overview of technologies for immobilization of enzymes and surface analysis techniques for

immobilized enzymes. *Biotechnol Biotechnol Equip, 29*(2), 205-220. doi:10.1080/13102818.2015.1008192

NIH. (2018). Lactose Intolerance. *Genetics Home Reference*. Retrieved from https://ghr.nlm.nih.gov/condition/lactose-intolerance#statistics

Schoevaart, R., Wolbers, M. W., Golubovic, M., Ottens, M., Kieboom, A. P., van Rantwijk, F., . . . Sheldon, R. A. (2004). Preparation, optimization, and structures of cross-linked enzyme aggregates (CLEAs). *Biotechnol Bioeng, 87*(6), 754-762. doi:10.1002/bit.20184

Sheldon, R. A. (2007). *Cross-linked enzyme aggregates (CLEAs): stable and recyclable biocatalysts*. Paper presented at the 7th International Conference on Protein Stabilization.

Talekar, S., Joshi, A., Joshi, G., Kamat, P., Haripurkar, R., & Kambale, S. (2013). Parameters in preparation and characterization of cross linked enzyme aggregates (CLEAs). *RSC Advances, 3*(31). doi:10.1039/c3ra40818c

Velasco-Lozano, S., López-Gallego, F., Mateos-Díaz, J. C., & Favela-Torres, E. (2016). Cross-linked enzyme aggregates (CLEA) in enzyme improvement – a review. *Biocatalysis, 1*(1). doi:10.1515/boca-2015-0012

Wang, M., Qi, W., Jia, C., Ren, Y., Su, R., & He, Z. (2011). Enhancement of activity of cross-linked enzyme aggregates by a sugar-assisted precipitation strategy: technical development and molecular mechanism. *J Biotechnol, 156*(1), 30-38. doi:10.1016/j.jbiotec.2011.08.002